Chapter 4
Applications of Molecular Markers and DNA Sequences in Identifying Fungal Pathogens of Cool Season Grain Legumes

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Abstract Molecular techniques have now been widely applied in many disciplines of biological sciences including fungal identification in microbial ecology and in plant pathology. In plant pathology, it is now common to use molecular techniques to identify and study plant pathogens of many agronomical and horticultural crops including cool season grain legume crops. In this chapter, we present two examples in which molecular techniques have been applied in order to identify and investigate multiple fungal pathogens causing two important diseases of chickpea and lentil. In each case, molecular techniques improved over traditional morphological identification and allowed timely and unambiguous identification of fungal pathogens. The first example involves identification of two *Sclerotinia* species (*S. sclerotiorum* and *S. trifoliorum*) causing stem rot of chickpea. Traditional method requires induction of carpogenic germination and observation of dimorphic ascospores in *S. trifoliorum*, which takes up to eight weeks. Taking advantage of the group I introns present in the nuclear small subunit rDNA of *S. trifoliorum* but absent in the same DNA region of *S. sclerotiorum*, a simple PCR amplification of the targeted DNA region allowed timely and reliable differentiation and identification of the species. The second example is of powdery mildew of lentil. Identification of powdery mildew fungi requires observing the teleomorphic (sexual) state of the pathogens, but this is not always available. In studying lentil powdery mildew in the US Pacific Northwest, we found that the powdery mildew on lentil does not fit previously reported species (*Erysiphe pisi* and *E. diffusa*). Further investigation confirmed that the lentil powdery mildew in the US is *E. trifolii*, a new pathogen of lentil. This discovery was mainly based on the rDNA ITS sequences and further confirmed by morphological and pathogenicity

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studies. These two examples demonstrate the important role of modern molecular techniques in solving practical agricultural problems. The ITS and adjacent rDNA could be ideal target regions for developing DNA barcodes for identifying these and related fungal species.

4.1 Introduction

Cool season grain legumes (chickpea, *Cicer arietinum*; faba bean, *Vicia faba*; lentil, *Lens culinaris*, and pea, *Pisum sativum*) are important crops worldwide. They are staple food crops in West Asian and North African countries and are important rotational and specialty crops in developed nations. Fungal diseases are important constraints in grain legume productions. Accurate identification of the fungal pathogens is in many cases a prerequisite for effective management of the diseases they cause and for ecological and population genetics studies. However, many fungal species are similar morphologically, and accurate species identification can be difficult. With current advances in biotechnology, molecular genetic markers have been employed for rapid identification of different kinds of fungi (White et al. 1990; Lieckfeldt and Seifert 2000; Njambere et al. 2008; Attanayake et al. 2009). The development of gene-specific primers for PCR amplification (White et al. 1990) has facilitated systematic studies, and the detection and identification of fungal pathogens. The internal transcribed spacer (ITS) region of nuclear ribosomal DNA has generally been considered a convenient marker for molecular identification of fungi at species level because of its conserved feature within species and multi-copy number per genome (Sanchez-Ballesteros et al. 2000). Henry et al. (2000) identified the fungus *Aspergillus* at species level and differentiated it from other true pathogenic and opportunistic molds using ITS 1 and ITS 2, allowing for early diagnosis and screening of effective antifungal agents for patients. Schneider et al. (1997) developed a method for detection of *Rhizoctonia solani* isolates, pathogenic and nonpathogenic to tulips, using ITS rDNA sequences, and they could further identify various anastomosis groups. Recent advancement in identifying fungal species using DNA markers is to develop DNA barcodes using species-specific oligonucleotides that are diagnostic of targeted species (Druzhinina et al. 2005). Such specific DNA regions need to be explored for different groups of fungi. In this chapter, we present two examples of applying molecular techniques in identifying fungal pathogens of cool season grain legumes.

4.2 Sclerotinia Stem Rot of Chickpea

Sclerotinia stem rot (Fig. 4.1a) is an important disease of chickpea under conducive environmental conditions and is caused by three species of *Sclerotinia*: *S. sclerotiorum*, *S. minor*, and *S. trifoliorum* (Bretag and Mebalds 1987). *Sclerotinia minor*

Fig. 4.1 Symptoms and signs of Sclerotinia stem rot of chickpea caused by *Sclerotinia trifoliorum* (**a**), and powdery mildew of lentil caused by *Erysiphe trifolii* (**b**)

can be easily differentiated from the other two species based on its numerous, scattered small-sized sclerotia in culture and in the field. Morphological difference between *S. sclerotiorum* and *S. trifoliorum* is subtle. The ultimate differentiation between *S. sclerotiorum* and *S. trifoliorum* requires observation of ascospore morphology which entails carpogenic germination of sclerotia. Ascospores of *S. trifoliorum* show spore-size dimorphism (two different-sized ascospores within a single ascus), whereas ascospores of *S. sclerotiorum* show no dimorphism (Kohn 1979; Uhm and Fujii 1983a, b). Induction of carpogenic germination of sclerotia of *Sclerotinia* spp. is a time-consuming process, and may take up to several months. To further complicate the matter, some isolates of *S. trifoliorum* are heterothallic and require mating with a compatible strain for carpogenic germination and ascospore production (Uhm and Fujii 1983a, b). Even though the process of identifying members of the genus *Sclerotinia* through sclerotia and other morphological characteristics has been refined over time (Kohn 1979; Rehnstrom and Free 1993), there are limitations to this approach. For instance, the differentiation of *S. trifoliorum* from *S. sclerotiorum* based on sclerotial characteristics is difficult because of instability of some sclerotia characteristics with subsequent sub-culturing (Cother 1977).

Therefore, to facilitate the separation of the two species, research efforts have been made in searching for molecular techniques that are reliable and convenient to use. Power et al. (2001) reported that *S. trifoliorum* contains group I introns in the nuclear small subunit rDNA, whereas *S. sclerotiorum* and *S. minor* do not contain any introns in the same DNA region. Molecular analysis of the ITS region can eliminate many of the problems associated with the morphological characters and culturing. Analysis of ITS sequence is usually applied to determine species identity (or sometimes higher taxonomic categories) and to identify and discriminate populations within a species. In the genus *Sclerotinia*, the ITS region is generally not sufficiently variable to distinguish within species diversity; however, the nuclear small subunit rDNA (nSSRrDNA) has been used for this type of study (Holst-Jensen et al. 1999; Power et al. 2001). In this study we explored the differences in the ITS and the nuclear small subunit regions of the rDNA between the two species causing *Sclerotinia* stem rot of chickpea.

4.2.1 DNA Isolation and ITS Sequence Analysis

DNA was isolated from mycelial mats or sclerotia using the standard extraction procedures such as the FastDNA® kit described by Chen et al. (1999). DNA quality was checked using agarose gel electrophoresis and quantified using the NanoDrop™ spectrophotometer (NanoDrop Technologies, LLC, Wilmington, Delaware, USA) and the concentration adjusted accordingly before PCR amplification. In our study PCR amplifications were conducted using primers ITS1 and ITS4 described by White et al. (1990). The PCR products were verified by agarose gel electrophoresis and purified for direct PCR sequencing using ABI PRISM 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA). Sometimes the PCR fragments are cloned before sequencing. Sequences were determined on both strands for each of the isolates and were aligned for comparison. Most sequence comparisons are carried out using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST) analysis which aligns two or more homologues to detect for presence of one or more ambiguous region within the segments under comparison. Using nine isolates from *S. slerotiorum* and *S. trifoliorum*, we amplified a 540 bp DNA fragment of the ITS region (Njambere et al. 2008). Sequence alignment among the nine isolates identified two single nucleotide polymorphic sites (SNPs) within this homologous region that differentiated the two groups of isolates. The two SNPs were located at position 120 (transversion T → G) and position 376 (transition T → C) of the amplicon. Three sequences of the isolates were deposited in the GenBank and assigned accession numbers EU082464, EU082465, and EU082466. BLASTn analysis of the ITS locus of some of the chickpea isolates (including EU082464, EU082465) displayed 100% homology to ITS locus of *S. trifoliorum* in the GenBank, whereas the ITS region of the other isolates (including EU082466) were identical to GenBank *S. sclerotiorum* isolates. These results therefore suggest that these two SNPs could be used as markers to separate *S. sclerotiorum* from *S. trifoliorum*. Although the ITS sequences allowed differentiation between *S. sclerotiorum* and *S. trifoliorum*, this technique is not convenient for routine identification because it requires DNA sequencing.

4.2.2 Detection of Group I Introns

Group I introns are ribozymes (RNA enzymes) that catalyze chemical reactions, splicing themselves off of their precursors. Group I introns are widely distributed in bacteria, lower eukaryotes, and higher plants. They can be found in genes encoding for rRNA, mRNA, and tRNA, but seem only in rRNA genes in the nuclear genome of lower eukaryotes. No biological functions are known for the group I introns except for splicing themselves off the primary transcripts. Although group I introns are known to spread from location to location and from one organism to another in evolutionary time, they are quite stable and their locations are highly conserved. Thus, if differences in existence of group I introns are found between two species,

the introns provide convenient markers for separation of the species because they can be easily detected through PCR and agarose gel electrophoresis. That is the case for *Sclerotinia* spp.

It was reported by Power et al. (2001) that *S. trifoliorum* contains group I introns in the nuclear small subunit rDNA, whereas *S. sclerotiorum* as well as *S. minor* does not contain group I introns in the same DNA region. We applied this knowledge in identifying *S. trifoliorum* from twelve isolates collected from chickpea plants. PCR amplifications were done using primer pairs ITS5/ITS4 and NS3/NS6 (White et al. 1990) in an attempt to detect presence or absence of introns in the nuclear small subunit regions of the rDNA. The reaction conditions were identical to those described above for PCR amplification of the ITS region. One or more group I introns were detected in all isolates of *S. trifoliorum*, and no group I introns were observed at any isolates of *S. sclerotiorum* (Fig. 4.2). Amplification with PCR can facilitate detection of the group 1 introns using PCR primer flanking the introns. Isolates with introns produce larger PCR products than isolates without introns, which can be easily detected using agarose gel electrophoresis (Fig. 4.2).

To be certain that the isolates harboring the group I introns are indeed *S. trifoliorum*, nine isolates were selected and induced to germinate carpogenically using a method as previously described (Njambere et al. 2008). For the isolates that germinated carpogenically, all the isolates that harbored introns in the rDNA region produced dimorphic ascospores, the ultimate criterion of identifying *S. trifoliorum* (Fig. 4.3). These confirmatory tests suggest that the group I introns in the rDNA region could be used for a quick and accurate identification of *S. trifoliorum* at

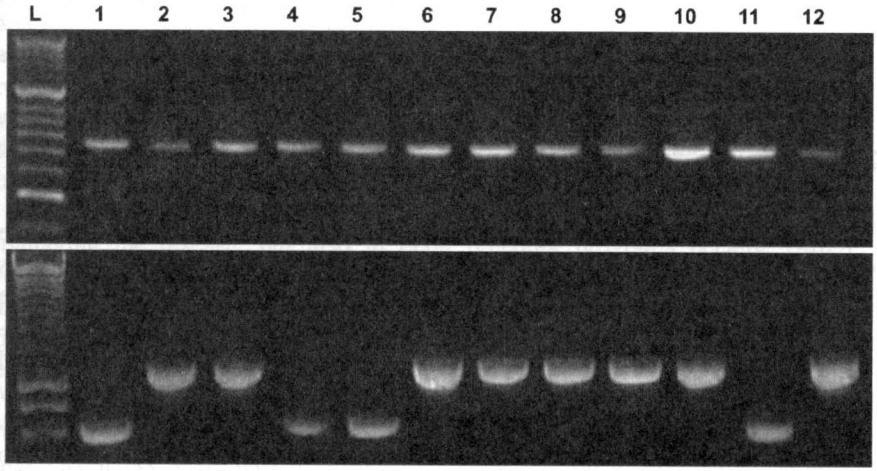

Fig. 4.2 Agarose gels of PCR amplification of the ITS and the nuclear small subunit rDNA regions of *Sclerotinia* spp. PCR products with primer pairs ITS1 and ITS4 (*top*) are monomorphic in size (no introns), whereas PCR products with primers ITS4 and ITS5 (*bottom*) are polymorphic in size (due to presence of introns). The lanes 1, 4, 5, and 11: *S. sclerotiorum* isolates (without introns); Lanes 2, 3, 6, 7, 8, 9, 10, and 12: *S. trifoliorum* (with introns)

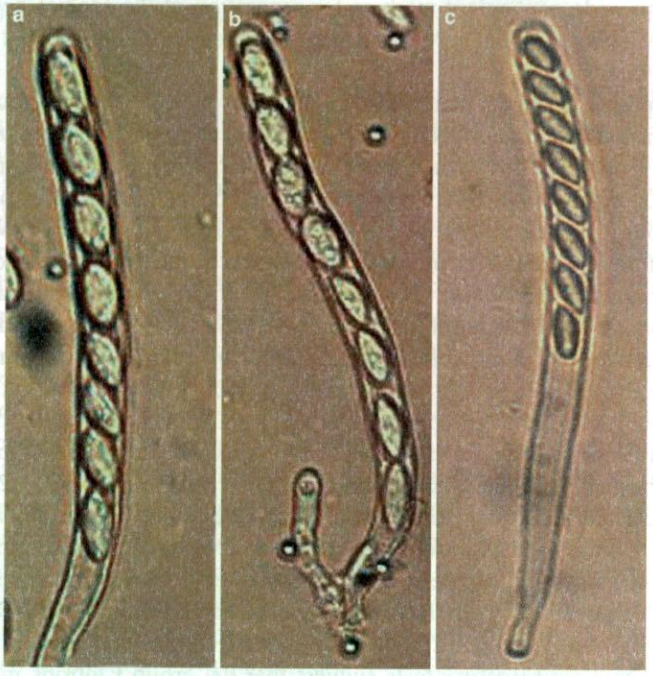

Fig. 4.3 Ascospore morphology of *Sclerotinia trifolorium* (**a** and **b**) showing size dimorphism, and of *S. sclerotiorum* (**c**) showing no size dimorphism

species level. We have employed this technique in identifying more than 100 isolates of *S. trifoliorum* for population genetic studies.

4.3 Powdery Mildew of Lentil

Powdery mildew is a plant disease caused by many different species of fungi in the order Erysiphales (Glawe 2008). The disease occurs in a wide range of plants. Its symptoms are very distinctive, powdery like spots on leaves and stems. The disease can reduce the yield and quality of many crops and commercial values of ornamental plants. In the field crop lentil, it can be a severe disease on certain cultivars and in some parts of the world, particularly in India during January to February (Agrawal and Prasad 1997). Although lentil is a field crop, breeding materials and many experimental plants are produced in greenhouses. Powdery mildew is a persistent disease problem of lentil plants in greenhouses (Beniwal et al. 1993), and poses a threat to precious breeding materials such as F1 plants. Infections by powdery mildews typically result in small white colonies on leaf surfaces (Fig. 4.1b). Lesions expand to cover entire leaf surfaces and pods. Mycelial growth and conidial production can be especially extensive at flowering. In case of severe

infections, leaves become chlorotic, then curled and necrotic prior to abscission. Yield decline may result and plants sometimes die (Agrawal and Prasad 1997).

Even though powdery mildew symptoms are easily recognized, identification of the species that causes the disease could be problematic (Glawe 2008). Knowing the species identity is important in devising management strategies as different species have different host ranges and different life histories. Identification of powdery mildew fungi relies on morphology of reproductive structures. Powdery mildews reproduce sexually by forming sexual structure chasmothecia (teleomorph) and asexually through conidia (anamorph). Traditional belief is that morphology of teleomorphs is more reliable than morphology of anamorphs. Taxonomy of powdery mildews of legumes is traditionally based on a few teleomorphic features, including chasmothecial appendage morphology (Braun 1995; Braun 1987) and host range. Powdery mildew pathogens that produce chasmothecia with multiple asci and dichotomously branched chasmothecial appendages were grouped into the genus *Microsphaera*, while otherwise similar, mycelioid appendage-bearing species were classified within the genus *Erysiphe* (Braun 1987).

However, recent phylogenetic studies of powdery mildew fungi using ribosomal DNA sequences demonstrated that anamorphic features are more indicative of phylogenetic lineages than are teleomorphic features, and that anamorphic characters are of utility in species determination (Braun and Takamatsu 2000; Cunnington et al. 2003; Glawe 2008). Chasmothecial appendages traditionally used to distinguish genera are now used to distinguish species (Braun and Takamatsu 2000). However, teleomorphic state is not always available and most of the time it forms when plants are senescent late in the growing season or does not form at all. It prevents timely detection and identification of the pathogen species. Even though abundant conidia are produced early in the disease development, there are only few anamorphic characters available (such as morphology and dimensions of conidia and conidiophores) to describe species and most of them overlap among closely related species. For example, conidia shape and sizes of *E. pisi* and *E. trifolii* are very similar and overlap considerably. Likewise, it is not reliable to use host ranges to identify powdery mildew species because many of them have broad and overlapping host ranges (Amano 1986).

Accurate determination of the pathogen species is very important not only for managing the disease, but also in plant breeding programs because different resistance genes may confer resistance to different pathogen species (Epinat et al. 1993). In some instances several powdery mildew species have been reported to occur together on the same host (Epinat et al. 1993; Glawe et al. 2004; Mmbaga et al. 2004).

Recent advances in molecular techniques have made it possible to investigate the species level identification of lentil powdery mildew pathogens. Use of molecular characters, especially ITS sequence data, has given promising results for species determination in some powdery mildews (Braun and Takamatsu 2000; Cunnington et al. 2003; Mmbaga et al. 2004; Takamatsu et al. 2002).

Powdery mildew of lentil is reported to be caused by two *Erysiphe* species, *E. pisi* (Amano 1986), a common pathogen of pea, and *E. diffusa* (Banniza et al. 2004), a

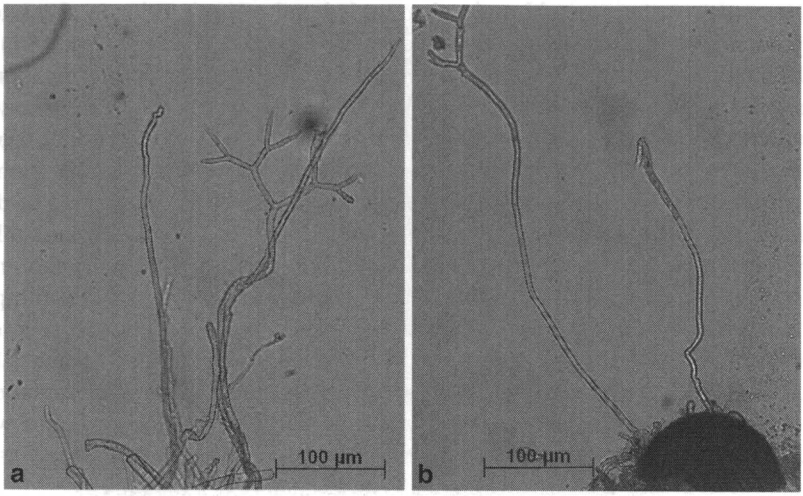

Fig. 4.4 Chasmothecium and its appendages of *E. trifolii* formed on an infected lentil plant. Highly branched chasmothecial appendages (**a**) and long flexuous nature of the chasmothecial appendages (**b**)

pathogen of soybean. The two species differ in conidia sizes. *E. pisi* produces conidia larger than those of *E. diffusa*. The major difference between the two species is that *E. diffusa* produces chasmothecial appendage with highly branched apices, whereas *E. pisi* produces mycelioid appendages. Powdery mildew is a frequent and serious disease of lentil plants in our greenhouses, but the species identity is not known. We observed that the conidia sizes larger than those described for *E. diffusa*. However, it produced chasmothecial appendages with regularly branched apices (Fig. 4.4), raising the possibility that it could be *E. diffusa*. The contradiction between the anamorphical characters and the teleomorphic characters gave confusion about the species identity. In order to ascertain the species identity of the powdery mildew fungus on lentil plants, we analyzed sequences of rDNA ITS region which led to the discovery of a new species of lentil powdery mildew.

4.3.1 Sample Collection and DNA Sequencing

Four samples of powdery mildews were collected from three different greenhouses over a 3-year period and an additional sample from the field was included in this study. Because *E. diffusa* is also a suspect species, a sample of *E. diffusa* from wild soybean (*Glycine* spp., kindly provided by Dr. Randall Nelson of USDA ARS, Urbana, Illinois, USA) was also included for comparison. Total DNA was isolated from conidia and/or mycelia from infected lentil plants using FastDNA® kit described by Chen et al. (1999). PCR amplification of the ITS region from each sample was performed using the primers ITS1 and ITS4 (White et al. 1990), or

Erysiphe- specific primers that we designed on the basis of conserved sequences of the ITS region of *Erysiphe* spp., EryF (5′TACAGAGTGCGAGGCTCAGTCG3′) and EryR (5′GGTCAACCTGTGATCCATGTGACTGG3′) (Attanayake et al. 2009). Amplified DNA fragments were first cloned into plasmid pCR2.1TOPO (Invitrogen Crop, Calsbad, CA). Plasmids containing inserts were verified by restriction digestion. The inserts were sequenced from both strands using one of the six primers: EryF, EryR, ITS1, ITS4, M13F, and M13R at the Sequencing Core Facility of Washington State University.

4.3.2 Sequence Analysis

All the ITS sequences of lentil powdery mildews collected from greenhouses and the field used in this study were identical to one another, but they differed in 18 nucleotide positions from the sequence of *E. diffusa* from a wild soybean *Glycine* sp. (Fig. 4.5). Sequences were used in BLASTn searches against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) to identify the most similar sequences available in the database. The sequences in the GenBank that showed the highest similarity (one base-pair difference) to the lentil powdery mildew sequence were three identical sequences (AB079853 to AB079855) of *E. trifolii*-like *Oidium* sp. from Japan (Okamoto et al. 2002). The sequences in the GenBank that showed the next highest similarity (three base pair differences) were five identical sequences (e.g., AB015913 and AF298542) of *E. trifolii* (Cunnington et al. 2003; Matsuda et al. 2005; Takamatsu et al. 1999), and another sequence (AB015933) of *E. baeumleri* (Takamatsu et al. 1999). The ITS sequence of the powdery mildew sample from wild soybean was identical to deposited sequences of *E. diffusa* in the GenBank.

Sequence accessions with high similarity values to the sequences determined in this study were aligned using the ClustalW program and used in phylogenetic analysis using the DNA Parsimony program of the PHYLIP package at http://bioweb2.pasteur.fr/phylogeny/intro-en.html. Parsimony analysis produced one most parsimonious tree with 113 steps. The sequence of lentil powdery mildew formed a tight cluster (monophyletic group) with sequences of *Erysiphe baeumleri*, *E. trifolii*, and *E. trifolii*- like *Oidium* spp., and is distantly related to (paraphylectic) *E. diffusa*. Another powdery mildew sequence from wild soybean specimen, also incorporated in this study, formed a separate clade with *E. diffusa* sequences in the GenBank.

4.3.3 Species Confirmation

As *E. trifolii* is not previously reported to be a pathogen of lentil, we needed to ascertain that the powdery mildew fungus on lentil is indeed *E. trifolii* and that it is

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E.trifolii   GCCGACCCTCCCACCCGTGTCGATTTGTATCTTGTTGCTTTGGCGGGCCGGGCCGCGTCG 60
E.diffusa    GCCGACCCTCCCACCCGTGTCGATTTGTATCTTGTTGCTTTGGCGGGCCGGGCCGCGCTG 60
             ************************************************************  *

E.trifolii   TCGCTGTTCGCAAGGACCTGCGTCGGCCGCCCACC-GGTTTTGAACTGGAGCGCGCCCGC 119
E.diffusa    TTGCAGTCCGCATGGACATGCGTCGGCCGCCCCCCCGGTGTTCCACTGGAGCGCGCCCGC 120
             * ** ** **** **** *************** ** *** **  ****************

E.trifolii   CAAAGACCCAACCAAAACTCATGTTGTTTGTGTCGTCTCAGCTTTATTATGAAAATTGAT 179
E.diffusa    CAAAGACCCAACCAAAACTCATGTTGTTTGTATCGTCTCAGCTTTATTATGAAAATTGAT 180
             ******************************** ****************************

E.trifolii   AAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGA 239
E.diffusa    AAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGA 240
             ************************************************************

E.trifolii   TAAGTAATGTGAATTGCAGAATTTAGTGAATCATCGAATCTTTGAACGCACATTGCGCCC 299
E.diffusa    TAAGTAATGTGAATTGCAGAATTTAGTGAATCATCGAATCTTTGAACGCACATTGCGCCC 300
             ************************************************************

E.trifolii   CTTGGTATTCCGAGGGGCATGCCTGTTCGAGCGTCATAACACCCCCTCCAGCTGCCTTTG 359
E.diffusa    CTTGGTATTCCGAGGGGCATGCCTGTTCGAGCGTCATAACACCCCCTCCAGCTGCCATTG 360
             ************************************************************ ***

E.trifolii   TGTGGCTGCGGTGTTGGGGCACGTCGCGATGCGGCGGCCCTTAAAGACAGTGGCGGTCCC 419
E.diffusa    TGTGGCTGCGGTGTTGGGGCTCGTCGCGATGCGGCGGCCCTTAAAGACAGTGGCGGTTCC 420
             ******************** ********************************** **

E.trifolii   GGCGTGGGCTCTACGCGTAGTAACTTGCTTCTCGCGACAGAGTGACGACGGTGGCTTGCC 479
E.diffusa    GACGTGGGCTCTACGCGTAGTAACTTGCTTCTCGCGACAGAGTGACGACGGTGGCTTGCC 480
             * ************************************************************

E.trifolii   AGAACACCCCTCTTTTGCTCCAGTCACATGGATCACAGGTTGACC 524
E.diffusa    AGAACAACCCTCTTTTGCTCCAGTCACATGGATCACAGGTTGACC 525
             ****** ****************************************
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Fig. 4.5 Alignment of ITS sequences of *E. trifolii* and *E. diffusa* determined in this study. An asterisk indicates an identical base pair. There are 18 base-pair differences between the two sequences. The *E. trifolii* sequence is > 99% similar to previously deposited sequences of *E. trifolii* in GenBank, and the *E. diffusa* sequence is identical to previously deposited *E. diffusa* sequences in GenBank

pathogenic on lentil. Three experiments were carried out to confirm that the powdery mildew pathogen of lentil in the US is *E. trifolii*, and not *E. diffusa*. First, conidia of *E. trifolii* were collected from lentil and used in a detached leaf assay to determine the pathogenicity on lentil under controlled conditions. Second, an authentic species of *E. trifolii* was obtained and compared with the samples from lentil in the US. The experiment showed that *E. trifolii* does produce long flexuous chasmothecial appendages with regularly branched apices similar to lentil samples (Fig. 4.4a, b). Finally, as *E. diffusa* is a common pathogen of soybean, soybean genotypes "L84-2237" and "Harosoy" known to be susceptible to *E. diffusa* were inoculated with conidia of powdery mildew from lentil and grown side by side with infected lentil plants in the greenhouse. The lentil powdery mildew did not infect soybean plants during the entire life cycle of soybean. These evidences strongly support the conclusion that the powdery mildew pathogen found on lentil in US was *E. trifolii* (Attanayake et al. 2009).

Powdery mildews of plants in the Fabaceae are very complex and have begun to receive more and more attention. Further taxonomic studies are needed because *E. trifolii* has been regarded as a complex of similar species consisting of *E. trifolii*, *E. baeumleri* Magn., and *E. asteragali* DC. (Braun 1987). The nature of this complex needs to be verified.

4.4 Conclusions

Modern molecular techniques have been used for identifying fungi in a wide array of biological science disciplines. In this chapter, we presented two specific examples of how molecular techniques have helped solve practical problems in identifying fungal pathogens of cool season grain legumes. In one case, we used molecular markers (group I introns and ITS sequences) to differentiate *S. trifoliorum* from a more common and closely related species *S. sclerotiorum*. This technique of identification allowed us to determine the species identity without the time consuming process of inducing carpogenic germination and ascospore observation. This technique allowed us to identify more than 100 isolates for studies in population genetics of *S. trifoliorum*. In the second example, using rDNA ITS sequences we were able to identify a new pathogen (*E. trifolii*) of powdery mildew of lentil. There were some ambiguities in determining the species because the morphology of teleomorph resembled a previously reported species (*E. diffusa*), but the anamorph is clearly different from *E. diffusa*. By comparing ITS sequences, examining an authentic specimen of *E. trifolii* and conducting pathogenicity test of a common host of *E. diffusa*, we unequivocally determined that the lentil powdery mildew was caused by *E. trifolii*. In doing so, we actually broadened the taxonomical concept of the species *E. trifolii* to include regularly branched chasmothecial appendages. Using these two examples, we have shown that modern molecular technology plays an important role and has gained increasing widespread applications in solving practical problems in agriculture. Furthermore, similar to what was found in species of *Trichoderma* and *Hypocrea* (Druzhinina et al. 2005), our results showed that the ITS region and the adjacent rDNA could be ideal candidate DNA regions used for developing DNA barcodes for identifying these and related fungal species.

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